Preface

Adrenergic receptors are important modulators in the sympathetic control of various metabolic processes in the central and peripheral nervous systems. These receptors are localized at multiple sites throughout the central nervous system (CNS) and serve as important regulators of CNS-mediated behavior and neural functions, including mood, memory, neuroendocrine control, and stimulation of autonomic function.

Adrenergic Receptor Protocols consists of 35 chapters dealing with various aspects of adrenergic receptor analyses, including the use of genetic, RNA, protein expression, transactivator, second messenger, immunocytochemical, electrophysiological, transgenic, and *in situ* hybridization approaches. This volume details the use of various methods to examine the adrenergic receptor system, using aspects of the genetic flow of information as a guide (DNA→ RNA → transactivator → protein expression → second messenger analyses → cellular analyses → transgenic whole animal approaches).

Adrenergic Receptor Protocols displays step-by-step methods for successful replication of experimental procedures, and would be useful for both experienced investigators and newcomers in the field, including those beginning graduate study or undergoing postdoctoral training. The Notes section contained in each chapter provides valuable troubleshooting guides to help develop working protocols for your laboratory. With *Adrenergic Receptor Protocols*, it has been my intent to develop a comprehensive collection of modern molecular methods for analyzing adrenergic receptors.

I would like to thank the many chapter authors for their contributions. They are all experts in various aspects of adrenergic receptors, and I appreciate their efforts and hard work in developing comprehensive chapters. As Volume Editor, it was a privilege to preview the development of *Adrenergic Receptor Protocols*, and to acquire insight on the various methodological approaches from different contributors. I would like to thank Professor John Walker, Series Editor for *Methods in Molecular Biology*, for his guidance and help in the development of this volume, and Thomas Lanigan, President of Humana Press. I would also like to thank Carol Houser for her administrative assistance in the preparation of manuscripts, and for members of my labora-

tory group, who have been helpful in the early stages of identification of potential chapter topics and contributors. Special thanks are extended to my wife, Dr. Cindy Machida, and my daughter, Cerina, for their support during the long hours involved in the compilation and editing of this volume.

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Isolation of Adrenergic Receptor Genes

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1. Introduction

In order to isolate a single gene, phage or cosmid libraries can be screened by the conventional technique of hybridization as described by Sambrook et al. *(1)* using end-labeled oligonucleotide probes or gene-specific probes. The probes are labeled either by nick translation, end labeling, or random priming using radioactive or nonradioactive techniques. Newer methods that use polymerase chain reaction (PCR) to screen phage libraries have been described by Yu and Bloem *(2)*. Below, we describe a method for screening a cosmid library using PCR rather than a conventional colony hybridization technique. The methodology is based on a report by Takumi and Lodish *(3)*. The cosmid libraries produce transformed bacterial colonies containing large cosmid vectors that behave as plasmids. These plasmids can be extracted from the bacteria by standard techniques for plasmid isolation once the appropriate clone is selected.

2. Materials

2.1. Cosmid Genomic Library Screening

1. Cosmid genomic library (*see* **Subheading 3.1.2.** in Chapter 1).
2. Luria-Bertani (LB) media: Dissolve 10 g Bacto-tryptone, 5 g Bacto-yeast extract, and 10 g NaCl in 1 L distilled water. Final solution is adjusted to pH 7.0 with approx 200 μL of 5 N NaOH and is sterilized by autoclaving.
3. LB agar plates: 15 g Bacto-agar in 1 L of LB media, sterilized by autoclaving, cooled to 55°C, and 30–35 mL poured into an 85-mm Petri dish and cooled.
4. 10 mg/mL kanamycin in water, sterilized by filtration through a 0.22-μm filter and stored at −20°C.

5. LB/kanamycin plates: sterile kanamycin is added to 50–55ºC molten LB agar (**item 3** above) to a concentration of 25 µg/mL, mixed, and 30–35 mL of the molten agar poured into an 85-mm Petri dish and cooled.
6. Gene-specific sense primer (25 pmol/µL water) and gene-specific antisense primer (25 pmol/µL water): synthetic oligonucleotide primers designed by the investigator (*see* **Subheading 3.3.**).
7. 10X PCR buffer: 500 mM KCl and 100 mM Tris-HCl, pH 8.3 (supplied with *Taq* DNA polymerase).
8. 25 mM MgCl$_2$ in water (supplied with *Taq* DNA polymerase).
9. 10 mM dNTP mix: dATP, dCTP, dTTP, dGTP, each dissolved in water at a concentration of 10 mM.
10. *Taq* DNA polymerase (5 U/µL) (Perkin-Elmer Foster City, CA or other supplier of licensed *Taq* DNA polymerase).
11. PCR mix for one 50-µL reaction: 5 µL 10X PCR buffer, 4 µL 25 mM MgCl$_2$ (final 2 mM), 0.5 µL 25 pmol/µL gene-specific sense primer (final 0.25 pmol/µL), 0.5 µL 25 pmol/µL gene-specific antisense primer (final 0.25 pmol/µL), 1 µL dNTP mix, 0.25 µL *Taq* DNA polymerase (5 U/µL), and 38.75 µL sterile water.
12. 2% Agarose gel.
13. 10X TAE electrophoresis buffer: 0.40 M Tris-acetate and 10 mM disodium EDTA (TAE), 48.4 g Tris base, and 3.72 g disodium EDTA in 850 mL water are adjusted to pH 8.0 with 10.6 mL glacial acetic acid, and the final volume is increased to 1 L with water.
14. Sterile 96-well microtiter dishes.
15. Glycerol: sterilized.

2.2. Analysis and Sequencing

1. Restriction endonuclease enzymes.
2. Hybrid phage/plasmid vector: Bluescript II (Stratagene, La Jolla, CA), pUC 18/19 (Life Technologies, Gaithersburg, MD).
3. Sequencing primers.
4. DNA sequencing facility or DNA sequencing kit: Sequenase DNA polymerase Version 2.0 kit (Amersham Life Sciences, Arlington Heights, IL) or Thermo Sequenase cycle sequencing kit (Amersham Life Sciences).
5. Oligonucleotide synthesis facility.

2.3. Selection of Primers for PCR

1. Oligonucleotide synthesis facility.
2. Sequences of adrenergic receptor subtype genes.
3. Computer programs for analyzing primers: Oligo 5.0 (National Biosciences, Plymouth, MN) or Prime (Genetics Computer Group [GCG], Madison, WI).
4. Computer programs for comparing DNA sequences: Pileup program (GCG).
5. PCR enzymes and reagents.
6. Thermal cycler.

2.4. Reverse Transcription and Selection of Primers

1. Oligonucleotide synthesis facility.
2. Sequences of adrenergic receptor gene subtypes.
3. Computer programs for analyzing primers.
4. Computer programs for comparing DNA sequences.
5. Random hexamers, oligo dT$_{17}$ primers or gene-specific antisense primers.
6. Reverse transcriptase enzymes.
7. PCR enzymes and reagents.
8. Thermal cycler.

2.5. Cloning of PCR Products

1. Cloning vectors.
2. TA cloning kits: TA Cloning or TOPO-Cloning kit (Invitrogen, Carlsbad, CA).
3. Restriction endonucleases.
4. Supplies for bacterial plating.
5. Procedures and kits for plasmid isolation.

2.6. Rapid Amplification of cDNA Ends (RACE)

1. cDNA with 3′ antisense adapter primer (*see* **item 2**) and 5′ sense adapter primer (*see* **item 3**) on respective ends (*see* Chapter 1, **step 11** in **Subheading 3.2.1.**).
2. 3′ antisense adapter primer: TTCCGGAATTCAGCGGCCGC 25 µ*M* (*see* Chapter 1, **item 20** in **Subheading 2.2.1.**).
3. 5′ Sense adapter primer: GACTCGAGTCGACATCGAC 25 µ*M*: primer derived from 5′ sense primer without the oligo dC tail (*see* Chapter 1, **item 17** in **Subheading 2.2.1.**).
4. Gene-specific sense primer (25 pmol/µL in water) and gene-specific antisense primer (25 pmol/µL in water): synthetic oligonucleotide primers designed by the investigator based on the sequence of the gene-specific PCR product isolated in **step 4** of **Subheading 3.5.**
5. 10 m*M* dNTP mix.
6. 10X PCR buffer: 500 m*M* KCl and 100 m*M* Tris-HCl, pH 8.3 (Perkin-Elmer).
7. 25 m*M* MgCl$_2$ (supplied with *Taq* DNA polymerase).
8. *Taq* DNA polymerase (5 U/µL) Perkin-Elmer or other supplier of licensed *Taq* DNA polymerase.
9. 100-Fold dilution of amplified cDNA from above (10–100 ng/µL).
10. Second internal gene-specific sense primer (25 pmol/µL in water) and second internal gene-specific antisense primer (25 pmol/µL in water): nested PCR synthetic oligonucleotide primers designed by the investigator based on the sequence of the gene-specific PCR product isolated in **step 4** of **Subheading 3.5.**, and internal to the gene-specific sense and antisense primers in **item 4** above.

3. Methods

3.1. Cosmid Genomic Library Screening

1. Titer the cosmid library by making the appropriate serial dilutions in cold LB/
   kanamycin (25 µg/mL) media and plating the dilutions on LB agar/kanamycin
   (25 µg/mL) plates. The plates are incubated overnight at 37°C and the colony-
   forming units/mL (CFU/mL) are determined (*see* **Note 1**).
2. Mix the cosmid library, and remove 16 aliquots such that each aliquot contains
   1×10^5 colonies/1 mL. Place the aliquots in separate microcentrifuge tubes.
3. Prepare a PCR master mix for a fraction more than the number of reactions
   (16 reactions and 2 controls) actually required (i.e., 18.5 reactions), or 92.5 µL
   10X PCR buffer, 74 µL MgCl$_2$, 9.25 µL gene-specific sense primer, 9.25 µL
   gene-specific antisense primer, 18.5 µL dNTP mix, 4.6 µL *Taq* DNA polymerase,
   and 716.9 µL sterile water (*see* **Note 2**).
4. After mixing the master mix, distribute 49 µL into 18 tubes. Place 1 µL from
   each of the 16 aliquots of the library into 16 of the tubes containing the master
   mix. Add 1 µL of water to 49 µL of master mix of the 17th tube, thus providing a
   negative control or no DNA template. Use the 18th tube as the positive control by
   adding the appropriate nanogram amount of genomic DNA in 1–49 µL of
   master mix.
5. Amplify the 1 µL aliquots of the library and the two controls according to previ-
   ously determined PCR conditions for approx 35 cycles (*see* **Notes 3** and **4**).
6. Electrophorese the PCR products through a 2% agarose gel containing 0.5 µg/mL
   ethidium bromide, and visualize with UV light.
7. Dilute the specific library pools or aliquots of cosmid that give a PCR product of
   the appropriate size to a concentration of about 30,000 clones/mL with LB/
   kanamycin media, or increase the volume of the aliquot by 3.3 vol. Aliquot 100 µL
   of each dilution (3000 colonies/well) into wells of a 96-well microtiter dish (*see*
   Note 5).
8. Pool the rows and columns of the above dilutions in the following way: combine
   10 µL of each well in the column into a microcentrifuge tube to give a final
   volume of 120 µL from the columns; combine 10 µL from each well in the rows
   into a microcentrifuge tube to give a final volume of 80 µL for the rows.
9. Remove 2.5 µL from each of the tubes containing pools of the respective col-
   umns and rows, and amplify the diluted cosmids using PCR master mixes and
   positive and negative controls as in **steps 3** and **4**.
10. Analyze the amplified products by gel electrophoresis to identify the tube from
    the pooled columns and the tube from the pooled rows that demonstrate the pres-
    ence of the appropriate PCR product. The well that intersects between a positive
    pooled column and a positive pooled row is indicative of a positive clone being
    present in the 30,000 clones/mL diluted wells.
11. Dilute this positive aliquot to 300 colonies/mL by increasing the volume 100-
    fold with the addition of approx 10 mL with LB/kanamycin media.

12. Aliquot 100 µL (30 colonies) of the diluted mixture into additional 96-well microtiter dishes.
13. Repeat **steps 7–9** above to identify a positive clone.
14. Plate the entire volume of the well with the positively identified clone on LB agar/kanamycin plates.
15. Toothpick each colony into individual wells of the 96-well microtiter dishes containing 100 µL of LB/kanamycin media, and grow for 6–8 h.
16. Pool and PCR 1-µL aliquots to identify positive clones as in **steps 7–9** above.
17. Grow any individual colonies that amplify the apparent gene-specific PCR product in 5 mL LB/kanamycin cultures for 6–8 h at 37°C with continuous shaking (*see* **Note 6**).
18. Add 15% (v/v) glycerol to 250 µL of the culture. Mix and freeze at −70°C for long-term storage.
19. Take the remainder of the culture and extract the cosmid from the bacteria using a standard alkaline lysis/phenol-chloroform extraction, small-scale plasmid DNA procedure (*see* **Note 7**).
20. Standard restriction digestion techniques are then used to digest 1 µg of the cosmid DNA with *Not*I, and analyze the digestion products by electrophoresis on a 0.8–1.0% agarose gel (*see* **Note 8**).

3.2. Analysis and Sequencing

1. The genomic insert should be restriction-mapped *(4)*, and the genomic DNA fragment that contains the coding sequence should be determined by Southern analysis using a gene-specific cDNA probe *(1)*. The relevant restriction-digested genomic fragments should be subcloned into a hybrid phage/plasmid vector such as Bluescript II (Stratagene) or pUC18/19 (Life Technologies).
2. Both strands of the recombinant plasmid inserts should be sequenced. A final sequence of the contiguous cosmid insert will be determined based on the restriction map and sequence of the restriction fragments.
3. If a sequencing facility is available, the plasmids can be readily sequenced using cycle sequencing techniques and fluorescence-based dideoxynucleotides. About 500 bp of sequence are generated in a sequencing run from four separate fluorescent dideoxynucleotide tags in one lane of an acrylamide sequencing gel.
4. Sequencing primers may be either vector-specific sequences adjacent to the cloning site or homologous to the gene-specific sequence. These gene-specific sequences would be determined after sequencing runs using vector-specific sequences near the cloning site.
5. The complete cosmid insert can also be directly sequenced using internal primers from the original genomic PCR product (*see* **item 6** in **Subheading 2.1.**) to sequence the cosmid in both directions (5′ and 3′). The resultant sequence is then used to develop new primers for the next sequencing run.
6. If a sequencing facility is not readily available, then the single-stranded or double-

stranded dideoxy chain-termination methods can be performed *(5)* using a
Sequenase DNA polymerase Version 2.0 kit (Amersham Life Sciences) or a
Thermo Sequenase cycle sequencing kit (Amersham Life Sciences) (*see* **Note 9**).

3.3. Selection of Primers for PCR

The sequence of the oligonucleotides used for the PCR should be selected
based on the guidelines listed below.

1. Primers should span a region of DNA with less than a 60% average GC content
   (*see* **Note 10**).
2. The sense (upstream) and antisense (downstream) primers should not be comple-
   mentary to one another especially at the 3′ end. In addition, they should not be
   complementary internally (palindromes), such that the primer can fold back on
   itself (*see* **Note 11**).
3. Oligonucleotides can range from 18 to 40 nucleotides in length, but for most
   applications 18–24 bp are sufficient.
4. The sense and antisense primers should have approximately the same G + C con-
   tent (40–60%). The melting temperatures, T_ms, for each primer should be within
   1–2°C of each other (*see* **Note 12**).
5. The primer annealing temperature for PCR is approx 5°C lower than the T_m of
   the oligonucleotides (*see* **Note 13**).
6. The selection of primers from known sequences can be determined visually or
   with computer programs. Two such programs are Oligo 5.0 (National Bio-
   sciences, Plymouth, MN) or Prime from GCG (*see* **Note 14**).
7. Primers for amplifying DNA of more than 2 kb in length (long-distance PCR,
   LDPCR) are designed to have higher annealing temperatures to provide greater
   specificity. When amplifying with these primers, cosolvents are added to lower
   the DNA melting temperature. It is also important to make sure that the selected
   primers do not contain repetitive sequences (Alu sequences) (*see* **Note 15**).
8. Noncomplementary bases (extensions) can be added at the 5′ end of primers.
   These extensions may code for restriction sites or promoter sequences or other
   sequences that are useful for cloning the amplified product into a vector or in
   vitro synthesis of RNA. When adding extensions for restriction endonuclease
   recognition sequences two to three extra bases (G or C) should be added on the
   5′ end, so that the enzyme has enough room to recognize the restriction
   site. These extensions will not hinder the PCR unless these sequences are present
   within the DNA region to be amplified.
9. The nonspecific binding and extension of primers prior to the initial denaturation
   of the template during the first step of PCR can be significantly reduced by
   keeping the reaction mixes at 0°C before thermal cycling and using "hot-start"
   techniques (*see* **Note 16**).
10. New receptor subtypes or multiple subtypes from species, which have not been
    rigorously studied, can be determined by designing primers based on two con-
    sensus regions from all the members of the same families. However, these

consensus regions should distinguish the family of interest from other families (i.e., other G-protein receptor gene families). Thus, the sense primer is identical to the upstream consensus region, and the antisense primer is identical to the downstream consensus region. We have successfully used this approach to clone the gerbil α-AR and β-AR *(6,7)*.

11. Consideration should also be given to the development of primers that span introns if a cDNA preparation is used as a template. The genomic database should be used to locate introns and then consensus primers designed on either side of the intron. Both the β_3-AR and α_1-AR contain introns. The α_1-AR in particular has at least one large 20- to 30-kb intron within the sixth transmembrane domain. However, the α_2-AR, β_1-AR, and β_2-AR are intronless (*see* **Note 17**).

12. Primers for cloning subtype-specific adrenergic sequences should be designed based on the consensus sequences that distinguish one subtype from another and are poorly conserved between subtypes, such as the sequences within the third intracellular loop of adrenergic receptors. The sense primer then is on the 5′ end of the loop and the antisense primer is on the 3′ end of the loop.

13. Database resources and computer programs can be utilized to help in the design of consensus sequences. The GenBank database can be accessed through the NCBI program Entrez (www.ncbi.nlm.nih.gov/Entrez/nucleotide.html), and keywords can be entered under the nucleotide search program. This search will identify all the sequences and their accession numbers, including expressed sequence tags (EST) sequences (or randomly transcribed cDNA) that have been submitted to GenBank. One should be aware that more than one keyword might be necessary to find all the sequences (i.e., adrenergic vs adrenoceptor). Occasionally, sequences published before sequence submission to GenBank was common may not have been entered into GenBank.

14. The investigator should understand that the name of the subtype for α_1-AR, α_2-AR, and β_3-AR that is described with the accession number is not necessarily the correct or current subtype nomenclature (*see* **Tables 1–3** in Chapter 1). The subtype specificity of the sequences can be determined by performing a multiple alignment comparison of the cDNA sequences of only the coding regions. We have successfully used the Pileup program from GCG to distinguish between the subtypes for the α_2-AR. This program will line up all the input sequences (nucleotide as well as amino acid) and output these data as a file. It will also produce a denogram that will group the sequences according to similar homology (**Fig. 1**). However, this is not an evolutionary tree and the grouping is only based on the similarities of the respective sequences (*see* Chapter 3, **Subheading 3.2.3.**) (*see* **Note 18**).

15. Once consensus sequence regions have been defined based on both nucleotide comparisons and amino acid homologies, it is possible that even though there may be an exact match in the amino acid consensus sequences, the nucleotide sequence may vary, especially at the third base of the codon. Thus, it may be useful to synthesize degenerate primers to encompass all the possible primer variations. Degenerate bases may be chosen based on the bases specific for each
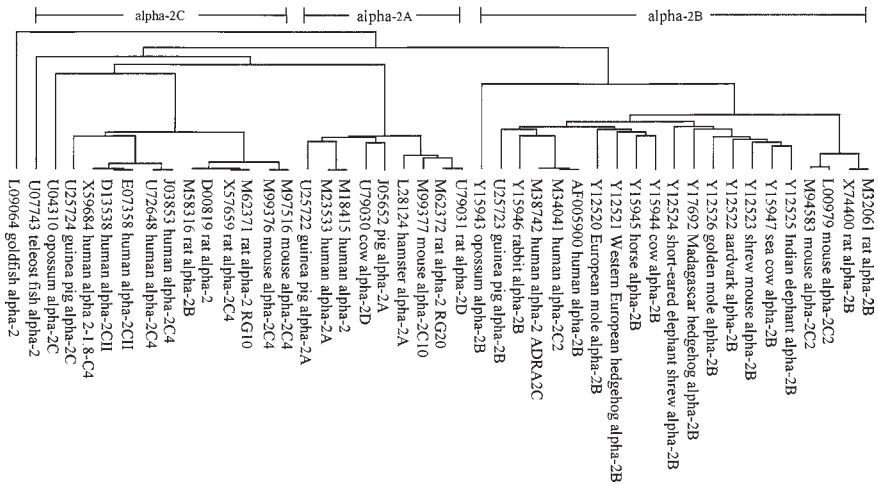
Fig. 1. Denogram of α_2-AR database sequences: Sequences were entered into the multiple sequence alignment Pileup program of GCG. Accession numbers and description of the sequences from GenBank are indicated. The three α_2-AR subtypes, α_{2A}-AR, α_{2B}-AR, and α_{2C}-AR, are indicated above the sequences. These assignments were made on the basis of the three major groupings of sequences as a result of the high identity of the nucleotide sequences within each group.

species or on all the possible nucleotide sequences for the amino acids at the primer site. However, the number of degenerate bases should be kept to a minimum. In particular, the base composition at the 3′ end should be conserved among all the species or subtypes (*see* **Note 19**).

3.4. Reverse Transcription and the Selection of Primers

Several different types of primers and reverse transcriptase (RT) enzymes can be used to form the first-strand or cDNA from either total or poly A$^+$ RNA templates (*see* **Notes 20** and **21**).

1. In general, random hexamers are used as primers when a transcript is very long, has a short poly A tail, and/or contains regions with a high % GC content, thus creating areas with significant secondary structures. Random hexamers are initially incubated at room temperature with RT to extend the primers such that they can anneal to the RNA at the higher RT incubation temperatures. Thus, these primers are not specific for any RNA species, either rRNA, tRNA, or mRNA, but the specificity for the amplified fragments can be achieved by using gene-specific primers at the higher annealing temperatures of PCR.
2. Oligo dT provides the next level of specificity by priming the synthesis of cDNA

predominantly from the poly A tail of mRNA. However, oligo dT may not tran-scribe efficiently if the RNA is partially degraded or the amplification target is significantly upstream of the poly A tail. The oligo dT primers are reverse-transcribed in the same manner as random hexamers, and the gene-specific prod-ucts are amplified at the more stringent conditions of PCR using gene-specific primers (*see* **Note 22**).

3. Finally, gene-specific primers that represent sequences from the antisense strand (or downstream primers) can be used for cDNA synthesis and can be designed based on consensus sequences. They are particularly useful for transcribing regions of mRNA that are distant from the poly A tail. We have successfully used this approach for amplifying adrenergic receptor subtypes from species, such as mouse or gerbil, and for quantifying RNA by competitive RT-PCR *(8)* (*see* **Notes 23** and **24**).

3.5. Cloning of PCR Products

PCR products have been historically difficult to clone because of the termi-nal transferase activity of *Taq* polymerase that adds single deoxynucleotides, in particular deoxyadenosine residues, to the 3′ end of the amplification prod-ucts. This creates an overhang rather than a blunt end and cannot be cloned into a blunt-ended vector.

1. PCR products can be cloned by engineering a restriction endonuclease recogni-tion sequence at the 5′ ends of PCR primers to create a compatible overhanging end with a cloning vector. Primers of this type are used in Chapter 1, **Subhead-ing 2.2.1., items 3** and **17**.
2. In other procedures, the products are blunt-ended, and cloned by filling the recessed ends with Klenow polymerase or by removing the extended bases with *Pyrococcus furiosus* (*Pfu*) DNA polymerase.
3. Finally, vectors with T overhangs can provide a sticky end for the A overhang in the PCR product in a procedure called TA cloning. This is one of the more popu-lar methods for cloning PCR products and is described by Trower *(9)* (*see* **Notes 25** and **26**).
4. The identity of the PCR products can be confirmed by sequencing, Southern hybridization with known probes, or restriction endonuclease analysis *(1)*.

3.6. Rapid Amplification of cDNA Ends (RACE)

After a product from RT-PCR of cDNA has been cloned and identified, that fragment can be used to screen a cDNA library in a similar manner to that described for screening a genomic library (*see* **Subheading 3.1.**). Alternatively, the small amount of sequence information derived from the RT-PCR fragment can be used to amplify sequences from both the 3′ end (3′ RACE) and 5′ end (5′ RACE) of the target message. The cDNA used above to construct the library (*see* Chapter 1, **step 11** in **Subheading 3.2.1.**) can be used as a template to

amplify the respective ends of a specific message. The adapter primers on the library cDNA provide a known primer sequence, or tag, on the 3′ or 5′ ends of all the cDNA species. Internal primers can be synthesized based on the sequence of the previously identified RT-PCR product and will serve as the gene-specific primers for the amplification reaction. Thus, the terminal adapter primers and gene-specific primers will be used during PCR to amplify two overlapping fragments of the target cDNA to complete the cDNA sequence. This methodology also has the potential to isolate products that are a result of alternative splicing of the same gene.

1. 3′ Ends are amplified by performing 30–35 cycles of PCR on the amplified cDNA in 50 µL as follows: 10–100 ng of amplified cDNA, 5 µL 10X PCR buffer, 4 µL $MgCl_2$ (final 2 mM), 0.5 µL gene-specific sense primer (final 0.25 pM/µL), 0.5 µL 3′ adapter primer (final 0.25 pM/µL), 1 µL dNTP mix (final 20 nM each), 0.25 µL *Taq* DNA polymerase, and sterile water to 50 µL. The PCR annealing temperature should be between 50 and 55°C, but these conditions will vary depending on the T_m of the gene-specific and adapter primers (*see* **Note 27**).
2. Analyze the amplified products by gel electrophoresis (*see* **Note 28**).
3. Perform a nested PCR reaction on the 3′-amplified product as follows: PCR 1 µL of the 3′-amplified product above (*see* **step 1**) with 5 µL 10X PCR buffer, 4 µL $MgCl_2$, 0.5 µL second internal gene-specific sense primer, 0.5 µL 3′ adapter primer, 1 µL dNTP mix, 0.25 µL *Taq* DNA polymerase, and sterile water to 50 µL (*see* **Note 29**).
4. Analyze the amplified products by gel electrophoresis and clone, and/or sequence the appropriate distinct band(s).
5. Amplify the 5′ end by repeating **steps 1** and **2** above using the gene-specific antisense primer in place of the gene-specific sense primer, and the 5′ sense adapter primer in place of the 3′ antisense adapter primer.
6. In a reaction similar to that in **step 3** above, perform a nested PCR on the 5′-amplified product from **step 5** using the 5′ sense adapter primer and the second internal gene-specific antisense primer.
7. Analyze the amplified products by gel electrophoresis and clone, and/or sequence any distinct band(s) (*see* **Note 30**).

4. Notes

1. Mix the library so that all the bacteria are suspended, and make three 10^{-2} serial dilutions and a final 10^{-1} dilution. Plate 100 µL from the final dilution (10^{-7} dilution) and the previous dilution (10^{-6} dilution) by spreading the aliquot with a bent glass rod that has been sterilized by immersion in 70% ethanol and flamed in a Bunsen burner. The rod is allowed to cool before spreading the bacteria. Ideally, dilutions that plate 100–300 CFU will allow a reasonably accurate determination of the titer.
2. The volume for the master mix should always be slightly larger than that required

for distribution to the tubes to eliminate the possibility of not having enough master mix for all the tubes.

3. The PCR reagent and cycling conditions for these reactions and the gene-specific primers (*see* **Subheading 3.3.**) should be predetermined using genomic DNA (100–500 ng/PCR reaction) as a template to amplify a specific portion of the gene. The ability of the primers to anneal to genomic DNA can be enhanced by denaturing the DNA (i.e., boiling followed by quick cooling on ice) before addition to the PCR mix. The identity of the PCR product should be confirmed (*see* **step 4** in **Subheading 3.5.**). Mg^{2+} concentrations for PCR may vary from 1 to 5 m*M*. Primers that amplify the 5′ end of the gene will select for clones with DNA near the transcription start site, whereas primers from the 3′ end of the gene will select clones nearer the 3′ untranslated region. Ideally, a cosmid library will allow the selection of the complete gene irrespective of the primers used. These reactions can also be performed in a 25-μL vol depending on the thermal cycler and test tube requirements.

4. There is no need to purify the DNA from the library, because the denaturing conditions at 95°C during PCR will lyse the cells to release the plasmid for amplification.

5. These plates can be stored for a day at 4°C after covering the dishes with parafilm. Adhesive acetate plate sealing material (Linbro, Flow Laboratories, McLean, VA) can also be used to cover the plates. The colonies should not be allowed to grow within the liquid media, since some clones will multiply at a faster rate than other clones and thus will potentially select against the target clone. For permanent storage, 15 μL of glycerol are added to each well and mixed. The plate is then stored at −70 or −80°C.

6. The yield of the cosmids will decrease with longer incubation times.

7. A typical procedure can be found in Sambrook et al. *(1)*. In addition, many plasmid isolation kits are also available from manufacturers and have been reviewed by Mack *(10)*.

8. Typical restriction digestion procedures can be found in Sambrook et al. *(1)*. A 7.9-kb vector and large (20–40 kb) insert should be observed. Smaller gene fragments might be observed if *Not*I cleaves within the gene insert.

9. Further information on cycle sequencing can be found in Brush *(11)*.

10. If the GC content of the PCR product is >60%, then cosolvents, such as 5–10% solutions of glycerol, dimethyl sulfoxide (DMSO), or formamide, may be required in the PCR reaction.

11. Primers that are complementary at the 3′ end will tend to form primer-dimers, which will compete with the genomic template.

12. The T_ms of the oligonucleotides can be approximated according to the following formula: $T_m = 4(G + C) + 2(A + T)$. Alternatively, the T_ms can be calculated using computer programs, such as Oligo 5.0 (National Biosciences) or Prime (GCG).

13. The exact annealing temperature for an optimum PCR can be experimentally modified by varying the annealing temperature in 2–5°C increments in either direction to establish primer conditions that are specific to the DNA. Magnesium

concentrations for PCR can also be varied between 1.5 and 5 m*M* to determine the optimum concentration.

14. The Prime program will design primers that span a selected region of the DNA sequence and will allow you to select the primer length, guanine-cytosine content, and T_m difference between your primers. In addition, the program will eliminate primers with internal and external complementarity.

15. The conditions for LDPCR can also be modified by lengthening the PCR extension times. In addition, small amounts of proofreading thermostable DNA polymerase enzymes (*Pfu*) are added to the *Taq* polymerase mix *(12)*.

16. "Hot start" can be carried out manually by heating all the PCR reagents, but *Taq* polymerase to 70–80°C, and then adding *Taq* polymerase at these higher temperatures. Immediately after the enzyme addition, the temperature is increased further to 95°C to commence denaturation and the thermal cycling procedure. Other techniques employ waxes that melt at 70–80°C and, thus, mix the aqueous enzyme mixture above the wax with the reagents below. An easier and more efficient procedure uses a *Taq* antibody, such as TaqStart (Clontech, Palo Alto, CA), which denatures and releases from the enzyme at 70–80°C as the PCR reagents are heated up to 95°C during the first denaturation step.

17. When cDNA is used as a template for intronless genes, untranscribed RNA templates should be included as controls to demonstrate the absence of genomic DNA.

18. The investigator can utilize the Pileup denogram to determine the subtypes of the sequences in the database based on the grouping of the sequences as a result of homology comparisons. This multisequence lineup is invaluable for determining consensus sequences between species by utilizing EST sequences and complete cDNA sequences.

19. This region of the primer will serve as an anchor for initiation of replication and extension by DNA polymerase, and heterogeneity in the primer at this position will result in too much mispriming. It is important to note that the annealing temperature and magnesium concentration may also need to be varied to account for the differences in bases within the degenerate primer subset. Conditions that might work for a known rat sequence may not be appropriate for the DNA of other species, depending on the degeneracy of the sequences. Further information on the synthesis of degenerate primers is reported by Preston *(13)*.

20. The reverse transcriptase enzymes most often used in these reactions are derived from Avian Myeloblastosis Virus (AMV) or Moloney Murine Leukemia Virus (M-MLV). AMV can reverse-transcribe at higher temperatures (48–55°C), thus eliminating secondary structure problems, and M-MLV can reverse-transcribe more efficiently and with less RNase H activity, but at lower temperatures (37–42°C). Other protocols are also available that utilize the same enzyme (*Thermus thermophilus*, *Tth*) for reverse transcription (70°C) as for PCR by changing the reaction conditions. A more complete description of reverse transcriptase enzymes and RT-PCR kits has been written by Wilkinson *(14)*.

21. In our laboratory, a typical reverse transcriptase reaction is performed in a 10-μL

vol with 5 mM MgCl$_2$, 1X PCR buffer, 1 mM dNTP, 1 U/μL RNase inhibitor, 2.5 U/μL M-MLV-RT, 2.5 μM antisense primers (or 2.5 μM random hexamer or 2.5 μM oligo dT primers) and ~1 μg total RNA. After incubating at 42°C for 50 min and then at 95°C for 5 min to denature the enzyme, the entire mixture is amplified by PCR in a 50-μL reaction with the appropriate sense or upstream primer and PCR reagents.

22. In comparison to random primers, oligo dT will tend to copy a greater proportion of the 3′ end of the mRNA, and random primers will transcribe more efficiently at the 5′ region of the mRNA.

23. The success of this approach is dependent on how specific the antisense primers are at the lower-temperature conditions of 42°C for cDNA synthesis by reverse transcriptase. Occasionally, we have found that the antisense primers are too non-specific at 42°C and will generate a RT-PCR product that appears to be of the expected size. However, when the product is sequenced, it is apparent that the antisense primer has served both as a sense and antisense primer in the PCR. Where the antisense cDNA primers give nonspecific products, then either more stringent PCR conditions could be employed or oligo dT and random hexamers could be used during reverse transcription to aid in the successful amplification of the gene-specific product.

24. In general, we have found that it is necessary to try each of the above primer approaches for cDNA synthesis. If the amplified product is of the expected size, it is useful to run PCR controls on this product in the presence of only the antisense primer and only the sense primer in PCR assays. Sequence analysis of the RT-PCR products should be confirmed by analyzing the products from three individual RT-PCR reactions to ensure that *Taq* polymerase has not introduced errors into the sequence.

25. In our laboratory, we routinely use the Original TA Cloning® kit or TOPO-Cloning® Kit from Invitrogen (Carlsbad, CA). These kits provide the materials for cloning the PCR product into a supplied linear vector. The insert can be conveniently excised from the vector with *Eco*RI or other convenient restriction endonuclease enzymes. Further, the vector contains M13 forward and reverse primers for sequencing. These kits are also designed to use PCR products directly from the PCR mix, from products isolated from low-melt gels, or extracted by other methods. Competent cells are also provided for transformation. Blue/white screening can be used to identify the recombinant plasmids. The colonies can also be picked with a toothpick, and a PCR reaction directly performed on the minute amount of bacteria using primers that flank the insert or gene-specific primers. The sizes of the products are then identified by gel electrophoreses *(15)*. DeFrancesco *(16)* gives a complete discussion of the PCR-based cloning kits that are available for cloning these products.

26. The investigator should keep in mind that PCR products generated by polymerases with 3′ to 5′ exonuclease activity, such as *Pfu*, will automatically create blunt ends that must be blunt-end-cloned or must be changed to single nucleotide overhangs in an extension reaction with *Taq* polymerase for TA cloning.

27. The DNA polymerase can also be mixed with proofreading enzymes to amplify potentially longer cDNA products with fewer sequencing errors.
28. Many times a smear will be observed for this initial reaction, and a second internal sense primer is then used to amplify the gene-specific product in a nested PCR reaction.
29. The amplified product from **Subheading 3.6., step 1** that is used as a template in this reaction can be removed directly from the PCR. Alternatively, the product can be used as the template if the DNA has been isolated from the gel. This can be done by either using a toothpick to add the PCR product directly from the agarose into the PCR mixture, or by cutting out the band containing the product isolation from a low-melt agarose gel and then melting the gel.
30. Variations of this procedure have also been described by Ausubel et al. *(17)* and Frohman *(18)*. In addition, several companies also supply kits for these procedures, such as the Marathon™ cDNA amplification kit from Clontech or 3′ RACE and 5′ RACE kits from Life Technologies.

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